CERTAIN CHEMICAL FACTORS INFLUENCING ARTIFICIAL ACTIVATION OF NEREIS EGGS [1, 2]

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Introduction

Stimulation must involve physicochemical changes within cells, and the nature of such changes has been the subject of much investigation, both experimental and speculative. The process of fertilization, and the closely related process of activation in artificial parthenogenesis, have attracted special attention; and evidence has been presented for a number of interesting interpretations of this type of activation. This report concerns a group of experiments indicating a peculiar relation of picric acid to the artificial activation of the eggs of *Nereis*. The proper interpretation of these experiments might contribute to the understanding of the stimulatory process. The experiments described developed from incidental observations in connection with heat-activation, during investigations concerned with the more general question of the mode of action of heat on protoplasm.

The peculiarities of heat-activation of the unfertilized *Nereis* egg were first described by Just (1915), who was able to interpret all his data in harmony with Lillie's "fertilizin" theories. In particular, Just attributed the gradual loss of sensitivity to heat, in eggs left standing in sea water, to the diffusion from them of some fertilizin-like substance, essential to the activating process. Heilbrunn (1925) took exception to this notion, in suggesting a "colloid chemical" interpretation of heat-parthenogenesis; he believed the decrease in sensitivity to heat might be due to the gradual loss of CO_2 from the medium, resulting in alkalinization of the intracellular fluid. Heilbrunn described three experiments in which the addition of 2–4 volumes per cent of n/10 HCl to old insensitive egg-suspensions restored their original sensitivity to heat.

To reveal a possible general relation between intracellular acidity or carbon dioxide concentration and the response of cells to increased temperatures, these three observations were extended. Heilbrunn's findings were in part confirmed; but with the accumulation of large numbers of experiments, considerable variation was encountered in the response of the heat-sensitivity of the eggs to increased CO_2 concentration through acidification of the sea water. Though such pronounced effects as described by Heilbrunn were often repeatable, as many batches of eggs seemed totally unresponsive to the same treatment. In the course of testing several organic acids in this connection, however, the anomalous properties of picric acid (2, 4, 6-trinitrophenol) came to light. Extension of these properties to processes of activation by means other than heat was then attempted.

[1] This study was carried out under the direction of Dr. L. V. Heilbrunn. I gratefully acknowledge his helpful suggestions throughout the investigations, and his valuable assistance in interpreting the results.

[2] A dissertation submitted to the faculty of the Department of Zoölogy of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Materials and Methods

Ripe females of the heteronereis form of *Nereis limbata* were captured between 8 and 10 p.m., as described by Lillie and Just (1913), and kept singly or in pairs in about 200 ml. of sea water in finger bowls overnight. These were kept cool either on a salt water bench in a stream of sea water, in a refrigerator, or in a room maintained at 15° C. The last was found by far the most satisfactory in maintaining the worms with eggs intact, with apparently no ill effects. A few worms shed their eggs during the night even at this reduced temperature; these were discarded.

All experiments were begun by the transfer of one or two *Nereis* to a Stender dish containing 25 ml. of sea water. The animals were cut transversely to release the eggs, and the carcasses were quickly removed. The eggs were then concentrated toward the center of the dish by gentle rotation. Either a small quantity of an especially dense suspension of eggs was then removed to another dish, or nearly all of the supernatant fluid was withdrawn by suction, and replaced by fresh sea water. All eggs were washed in this manner through at least another change of sea water, before use. In the earliest work, samples were always tested for normalcy by treatment with sperm from males caught in the same swarm. Only very rarely was any egg ever found which did not become normally fertilized, and all samples showed well over 99 per cent germinal vesicle breakdown. Since this dependability of *Nereis* eggs is well known, and since all danger of accidental contamination of eggs with sperm was to be avoided, no such tests were made in most of the later work. Experiments were always begun on the day following capture, so that the lapse of time between capture and the first treatment was never more than 20 hours, and was only rarely over 15 hours.

All transfers of eggs were made with ordinary medicine droppers. All treatments and exposures, unless otherwise indicated, were made in a volume of 25 ml.; the egg-suspensions were of such a density that, upon settling of the eggs to the bottom, no more than half, and usually much less, of the bottom of the container was covered with a single layer. Stender dishes of about 35 ml. capacity were used except for the exposures to high temperatures; the latter were carried out in 50 ml. beakers, in which the thermal insulation is much reduced. The beakers were immersed in a small deKhotinsky constant-temperature bath to a depth 2–3 mm. above the surface of the inside liquid. The temperature of the fluid within the beakers was brought to equilibrium (at slightly less than half a degree lower than the bath temperature) before the addition of 0.3–0.5 ml. of the egg-suspension. The activating temperature used varied between 33° and 35° C., as in Just's work (1915), but was held constant to within 0.1 of a degree for any single series of tests.

Since it soon became evident that the degree of stirring had a considerable effect on the response to heat, a standard policy in this regard was always followed: upon deposition of the eggs in the warm beakers, the pipette was filled and emptied ten times successively within 4–5 seconds. This was repeated 4 minutes after the beginning of the exposure; and the beaker was removed after 5 minutes of exposure, at which time a sample of 5–8 ml. was removed to a Syracuse watch glass. In some of the earlier work, the second stirring was performed at 15 minutes, the beakers removed at 20 minutes. This exposure, which is approximately Just's optimum, yielded a better percentage of swimmers, but the shorter exposure was found to

produce the maximal amount of germinal vesicle breakdown, and was much more convenient in extended series of tests. A few tests indicated that further stirring and longer exposures led to no increase in the percentage of activation. A further trial showed that the immediate removal to Syracuse dishes was not essential; when the beakers were allowed to cool of their own accord, the residual heat did not affect the percentage of activation.

For counts of activation, 5–8 ml. of each egg-suspension were examined in a Syracuse watch glass at a magnification of about 100 ×. In certain cases involving a doubtful response, compression of the eggs between a slide and coverslip, as suggested by Heilbrunn and Wilbur (1937), and a higher magnification were necessary. The counts were made on the basis of the breakdown of the germinal vesicle, a reaction which normally occurs soon after fertilization. Counts were begun at a minimum of 2 hours after the application of the treatment in question. The advantages of the nuclear criterion are its rapidity of onset, its definite character (ordinarily admitting of easy and certain classification in counting), and its ready susceptibility to quantitative expression; the criterion is well established in work on artificial activation of this form. However, the fact should not be overlooked that the mere breakdown of the germinal vesicle in response to stimulation is seldom followed by development even approaching the normal, and there is rarely any cleavage at all. Various types of monsters are produced, mostly of the type described as due to "differentiation without cell-division," common in annelids. All of the types of stimulation used were capable of producing at least a small percentage of swimming forms, though seldom was anything like a normal trochophore seen. All counts were of 100 or 200 eggs selected by random movement of the watch glass on the stage of the microscope.

Results

Upon standing in sea water, almost all batches of eggs showed a gradual loss of sensitivity to heat, as described by Just (1915); a few, however, showed a very definite increase in sensitivity, after washing and long standing. This might perhaps be attributable to the washing away of inhibitors in the body fluids (Just, 1915); but the most pronounced of these exceptions was in a special batch in which the eggs stood in a deep layer at the bottom of a narrow container. Thus the responsible factor may have been the high CO_2 tension, in accordance with Heilbrunn's views (1925). Of several organic acids tested, however, only picric acid produced a consistent and pronounced reversal of this loss of sensitivity to heat. After a batch of eggs had become nearly or quite heat-insensitive, a bath of 15 minutes or more in sea water to which picric acid had been added to a concentration of about M/1000 (pH 6.6) was sufficient to elicit a significant response to the subsequent heat treatment in sea water. Yet the presence of the acid in the heat-treated suspensions completely prevented the activation of the eggs; if a response was to be obtained, the eggs had to be transferred back to sea water for the heat treatment.

These aspects of the action of picric acid were then tested in connection with activating agents other than heat. The agents used were ultra-violet irradiation (Heilbrunn and Wilbur, 1937), mixtures of sea water and isotonic (0.53 M) KCl (Wilbur, 1939), and mixtures of sea water and isotonic (0.35 M) sodium citrate

(Wilbur, 1941). Mixtures of KCl or citrate with sea water are denoted after the terminology of Wilbur (1941); thus a mixture of one volume of isotonic citrate and four volumes of sea water is called a "20 per cent sodium citrate mixture."

Experiments showing inhibition by picric acid of various types of activation

Heat—Of 15 experiments on the effect of picric acid on the sensitivity of eggs to heat, only one was inconsistent with the thesis that the acid inhibits the heat-activation. In these experiments, M/1000 picric acid was used, made up in sea water. Eight experiments proved useless, as the control percentages were too low to test any possible inhibition by the acid; the heat-sensitivity of these eggs is notoriously very variable between batches from different animals. The average of the seven experiments in which over 10 per cent of the control eggs responded is included in Table I, and shows a marked inhibition of the response by picric acid.

TABLE I

Inhibition by picric acid of activation of Nereis eggs by various agents

Activating agent	No. of expts.	Per cent activation in absence of picric acid	In picric acid, M/1000	
			Per cent activation	Per cent with incipient activation*
Heat	7	57	4	0
KCl mixtures	18	96	4	51
Sodium citrate mixtures	19	99	23	28

* As described on p. 147.

KCl mixtures—In fourteen experiments in which eggs were left indefinitely in a 25 per cent KCl mixture, and four similar experiments with a 50 per cent KCl mixture, almost always there was nearly 100 per cent activation in the absence of picric acid. When the acid was added to a concentration of M/1000, such activation occurred in only one instance; this case was distinctly unusual, as 74 per cent of the eggs were activated. Table I includes the averages for these experiments. However, in only 5 of the 18 tests was the breakdown of the germinal vesicle completely prevented. In the others, ordinary methods of observation (at 100 × magnification) did not reveal any certain change in appearance from the germinal vesicle stage, but a distinct nuclear outline could not be made out in many eggs. Compression of the eggs and higher magnification were necessary in counting these batches; the criterion employed was the visibility of a definite interface between the spherical nucleus and the cytoplasm. In the absence of this interface, the germinal vesicle was said to be broken down, even though no real alteration in the appearance of the egg was evident; the average percentage of the eggs so classified is presented in the last column of Table I. In these cells, the central nuclear region remained clear, the granular cortical opacity was retained, the oil droplets remained discrete and failed to migrate as in the activated eggs. None of the eggs of this type ever developed to a motile condition, or cleaved, or differentiated in any way. The appearance was as though nuclear breakdown had just barely begun when inhibition set in.

Sodium citrate mixtures—Complete or nearly complete inhibition of activation by picric acid was found in 12 of 19 experiments with citrate mixtures of 10–25 per cent. Of the other seven, two showed effective inhibition beyond the earliest stages of nuclear breakdown, as with the KCl mixtures (last column of Table I); one showed only moderate inhibition; only 4 of the 19 failed to show any significant inhibition. The averages are included in Table I. In these experiments, as in those with the KCl mixtures, the eggs were left in the activating agents indefinitely; counts were made with the eggs still in the various mixtures.

Ultra-violet irradiation—Only in relation to activation by ultra-violet rays did picric acid fail to exhibit an inhibitory effect. The presence of the acid (M/1000) in the sea water bathing the eggs did effectively prevent their activation by irradiation, but this action cannot be attributed to the effect of the acid on the eggs. Reduction of the depth of the egg-suspension to under 0.5 mm., so that the eggs are barely covered, permitted of ready activation by the rays, even in the presence of picric acid. The apparent inhibition in deeper samples is due to the absorption of the rays by the acid; the absorption spectrum of picric acid and picrates in salt solutions near neutrality (Eisenbrand and v. Halban, 1930; v. Halban and Litmanowitsch, 1941) is such that in any appreciable depth and concentration the supernatant fluid would prevent most of the active radiation from reaching the eggs, which always settle to the bottom of the dish. This interpretation is corroborated by the fact that a shield of picric acid in a quartz dish prevents any effect of ultra-violet rays on an underlying suspension of eggs in sea water.

Fertilization by sperm—Normal fertilization is completely inhibited in the solutions of acid used for the experiments above (in the range of M/1000). Addition of alkali to pH 8.0 did not affect this inhibition of fertilization. However, the removal of normally fertilized eggs to picric acid solutions within five minutes after fertilization (whether or not such solutions were alkalinized) did not appear to interfere with the normal development of the embryos; excellent survival and differentiation were obtained in the acid. Nevertheless, such embryos exhibited one outstanding anomaly: failure of the normal coalescence of the oil droplets. The oil in embryos growing in picric acid remained scattered as numerous discrete droplets; while under normal conditions these soon merge to form only a few, almost always four. The usual localization of the oil by migration (and segregation in cleavage) was not, however, altered in the course of development in picric acid solutions.

Experiments showing synergism between various activators and the removal from picric acid to ordinary sea water

Heat—Over 50 experiments tested the effect of baths in picric acid prior to exposure to heat in sea water. These showed a pronounced enhancement of the effects of the heat after the acid bath; not one showed a greater activation in the sample from sea water than in that from the acid. This relation between heat and removal from picric acid baths is shown in Figure 1. The synergistic action is evident only following the shorter baths, up to about 6 hours; since, after longer exposures to the acid, the mere removal to sea water was in itself sufficient to activate many eggs. The broken line curve in Figure 1 is made up from the combined data of all experiments involving removal of eggs from picric acid to sea water without further treatment. The other two curves on the same figure, however,

cover data from paired samples of eggs, and compare the effects of heat on eggs previously bathed in picric acid (in sea water) and on eggs from the same source not so treated.

The synergistic action was evident over a wide range of concentration of picric acid: from 10^{-4} to just over 10^{-3} M. The effects increased with increasing concentration, but above M/1000 the results became less reliable, so that M/1000 was used regularly, and is the only concentration for which data are reported. It is evident

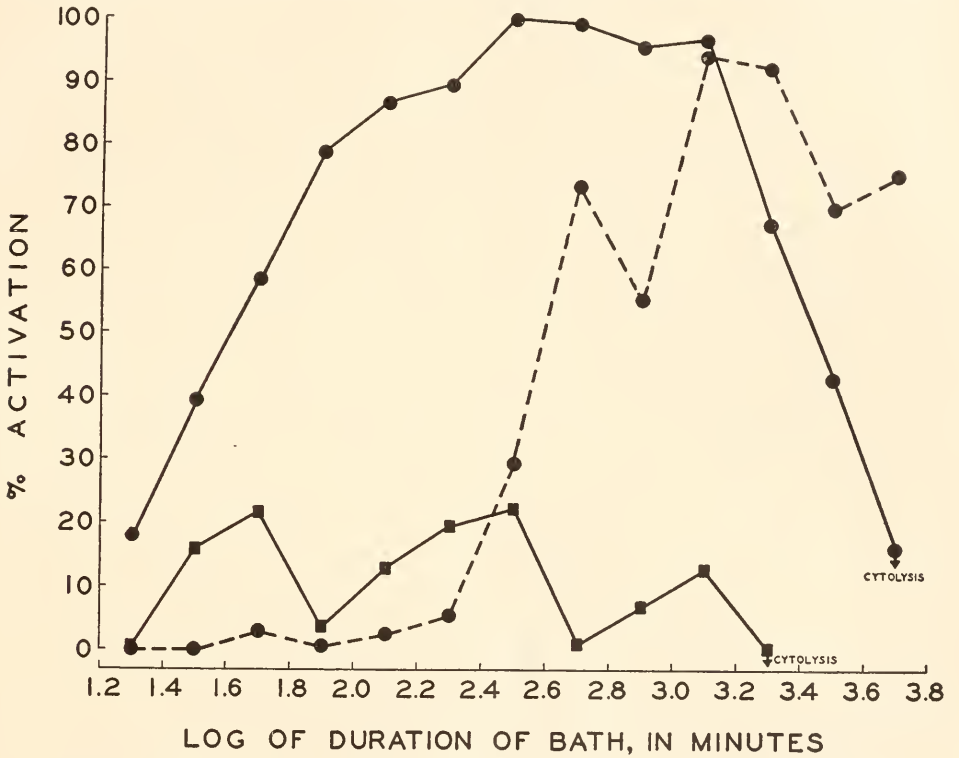

FIGURE 1. Relation of previous baths in picric acid to heat-activation of *Nereis* eggs.

Solid line connecting circular points—eggs heated after bath in M/1000 picric acid in sea water.

Solid line connecting square points—eggs heated after bath in sea water.

Broken line—eggs removed, unheated, from bath in M/1000 picric acid in sea water.

Each point is the average of all experiments performed in the logarithmic time interval denoted at the base-line. See text for further explanation.

from Figure 1 that the unfertilized eggs survived in the acid about twice as long as in sea water. Removal from sea water to the acid just prior to the expected onset of cytolysis (about 30 hours after removal from the animal) preserved the eggs as well as, but no better than, storage in the acid from the beginning.

KCl mixtures—The synergistic action of KCl mixtures and removal from picric acid to sea water was tested in 14 experiments, summarized in Figure 2(a). After 2–8 hours in the acid solutions, samples of eggs were removed to sea water and

to 5 per cent KCl mixtures; a control sample of the same batch of eggs kept in sea water was simultaneously exposed to the 5 per cent KCl mixture. This concentration of KCl is just below that necessary to induce regularly an appreciable percentage of response in ordinary eggs. Though the combined treatment was not in every case sufficient to activate the eggs, most experiments showed a pronounced synergism, and none showed a difference in the opposite direction. The response

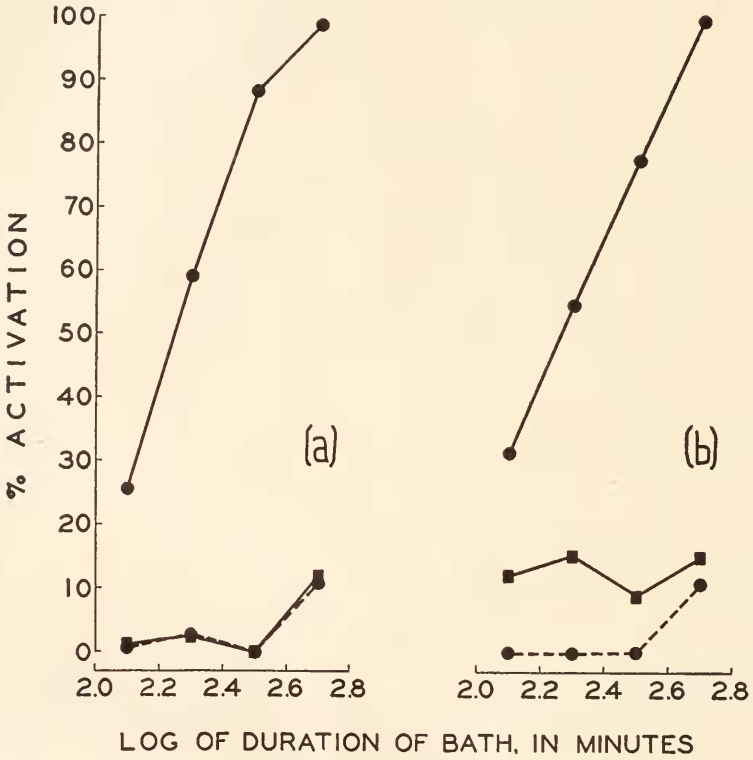

FIGURE 2. Relation of previous baths in picric acid to activation of *Nereis* eggs by (a) KCl mixtures, (b) sodium citrate mixtures.

Solid line connecting circular points—eggs treated after bath in M/1000 picric acid in sea water.

Solid line connecting square points—eggs treated after bath in sea water.

Broken line—eggs removed, untreated, from bath in M/1000 picric acid in sea water.

Each point is the average of all experiments performed in the logarithmic time interval denoted at the base-line. See text for further explanation.

of the eggs in these experiments, upon removal from picric acid baths to sea water, was somewhat less than average; thus the broken lines in the graphs in Figure 2 differ somewhat from the similar curve in Figure 1.

Sodium citrate mixtures—The same action was demonstrated with 10 per cent sodium citrate in place of the 5 per cent KCl mixture; 14 of 19 experiments showed a decided synergism. Five failed to show any significant difference between control and experimental. These failures were all among the shorter expo-

sures to the acid; the longer baths always resulted in increased sensitivity of the eggs to the citrate. This is illustrated clearly in Figure 2(b), which includes the data from all 19 experiments.

Ultra-violet irradiation—All attempts to show synergism between ultra-violet irradiation and removal from picric acid baths failed. A Uviarc mercury-vapor lamp, operating at 110 volts, 60 cycles, was used. The intensity of the radiation at the point at which the eggs were exposed was on the order of 6000 micro-watts per square centimeter.[3] Under such conditions, no significant differences could be found between the response to irradiation of eggs just removed from picric acid baths and those from sea water.

Discussion

The data illustrate three aspects of the action of picric acid in relation to activation of the eggs:

(1) in the presence of certain concentrations of picric acid, heat-activation and chemical activation are prevented;

(2) removal from the same concentrations of picric acid to sea water, after a short stay in the acid, acts synergistically with other activating agents in causing nuclear breakdown;

(3) removal from the acid to sea water after longer stays in the acid leads to activation without assistance from other agents.

That fertilization and maturation of marine eggs is inhibited by acids is a common observation (Clowes and Greisheimer, 1920; Smith and Clowes, 1924; Tyler and Schultz, 1932; Tyler and Scheer, 1937); so that the inhibition by picric acid of artificial activation is not surprising. Similarly, the preservation of the unfertilized egg in picric acid against cytolysis and death is in accordance with many observations of this action of acids; some treatments were reported far more effective in this respect than picric acid appeared to be (Carter, 1931; Just, 1920; Smith and Clowes, 1924; Tyler and Horowitz, 1937a; Tyler and Dessel, 1939). The suggestion has even been made (Tyler, Ricci, and Horowitz, 1938) that the greater life-span of eggs in alcohol, dextrose, anoxic media, etc. (Gorham and Tower, 1902; Loeb, 1902; Loeb and Lewis, 1902; Lillie, 1931; Whitaker, 1937), can be explained in each case by the production of acids. The only odd aspect of the action of picric acid in this regard is that eggs stored in it for some time are subsequently over-sensitive to stimulators, and eventually are activated merely by removal to sea water. This was observed after a stay in the acid of as much as 70 hours. This is entirely dissimilar to the acid activation of starfish eggs, as investigated extensively by Lillie (1926, 1927, 1934, 1941). Lillie's exposures were of only a few minutes' duration, and the eggs were visibly altered while in the acid; a slightly prolonged exposure destroyed the eggs altogether. In picric acid, however, the eggs remain apparently unchanged for days, but immediately respond when removed to sea water.

This fact leads to the postulate that picric acid may react with, or in some way inactivate, an activating agent produced within the egg. Above a certain concentration, this agent would lead to activation of the egg; in still greater concentration, or under other conditions, to cytolysis. This agent is apparently being constantly

[3] Thanks are due to Dr. A. C. Giese for this measurement.

produced, and either diffuses from the egg, or is gradually destroyed as it is produced. But when picric acid is present within the egg, this agent is retained by the acid in an inactive form; when the egg is removed to sea water, the picric acid diffuses away, in turn releasing any acid bound with the activating agent. Thus the inhibition is removed, so that there is a sudden release of the accumulated activator within the egg, causing a response if the accumulation has been great enough.

Such a suggestion is in harmony with the synergism found between other activating agents and the removal from picric acid after exposures of lesser duration, and with the temporal pattern of the development of this synergism, as shown in Figures 1 and 2. The activating agents may be supposed to act by accelerating the production of the hypothetical activating substance; subliminal doses of these agents may then produce enough of the substance so that the added quantity released from the picric acid suffices to produce the response. That a still greater concentration may lead to cytolysis is indicated by the fact that less activation, with considerable cytolysis, is found when eggs are heated after a very prolonged exposure to picric acid, than when they are simply removed from the acid at the same time to sea water, without heating (Figure 1).

TABLE II

Synergism between various activating agents in stimulation of Nereis eggs

Activating agents		No. of expts.	Per cent activation		
A	B		A alone	B alone	Both
Heat	Sodium citrate 5%	9	24	0	56
	10%	13	20	4	83
KCl 5%	Sodium citrate 6%	1	0	0	86
	8%	1	0	2	99
	10%	1	0	78	100
Heat, without usual stirring	Stirring	4	15	0	39

The synergistic action indicates that at least to some extent activation is brought about through the same channels by all four agents: heat, KCl, sodium citrate, and removal from picric acid to ordinary sea water. Added evidence in this direction was obtained in experiments showing pronounced synergistic action between heat and citrate mixtures, and between KCl mixtures and citrate mixtures (Table II). As previously mentioned, stirring during exposure to heat had a pronounced enhancing action on the stimulatory effect of the heat, but stirring did not appear to act similarly in connection with the chemical activators. Mathews (1901) reported that Loeb and Fischer had been able to activate *Nereis* eggs by mechanical agitation alone, but all attempts in this direction failed.

Attempts to show synergism between ultra-violet irradiation and sodium citrate mixtures or removal from picric acid all failed; this is in keeping with the failure of picric acid to inhibit activation by ultra-violet rays. This may indicate that the radiation acts through a different mechanism than that involved in stimulation with the other agents. But under the conditions of the experiments the duration of

the exposures to ultra-violet was on the order of 30–60 seconds, much less than with the other types of activation; this difference in the rate of activation may be the entire explanation for the non-conformance of the experiments with this type of activation.

Heilbrunn (1925), Heilbrunn and Wilbur (1937), and Wilbur (1939, 1941) have presented several lines of evidence indicating that the breakdown of the germinal vesicle in the *Nereis* egg involves a reaction of calcium ions with the colloids of the protoplasm, and an associated set of changes in viscosity. Heilbrunn proposed that stimulating agents act by freeing calcium ions from combination (with lipoprotein) in the cell cortex, so that the calcium may react with the inner protoplasm; this interpretation of stimulation has been applied not only to the eggs of *Nereis,* but to cells in general. If such a mechanism is actually involved in the response of the *Nereis* egg, it might be expected that a penetrating acid would inhibit activation. The picric acid might acidify the protoplasm to the extent that the amphoteric protein molecules would become predominantly cations, with less Ca-binding capacity than previously. This interpretation would perhaps also explain the activation found upon removal of eggs from picric acid baths to sea water; the calcium freed from the cortex by the acid could react with the cell interior upon removal of the acid. Thus the same agent would act, in a sense, both as activator and as anesthetic. A serious difficulty with this explanation of the data lies in the fact that the eggs must be left in the acid for several hours, if they are to respond upon removal to sea water. This would require the assumption that the liberation by the acid of calcium ions from the cortex is a very slow process; or else that the acid continues to accumulate within the egg over a period of hours, quickly rising to the inhibitory concentration, but only after hours attaining the concentration active on the cortex. Neither of these assumptions is impossible, but both are rather involved.

If the action of picric acid were due to this proposed effect on the Ca-binding properties of proteins, other acids might be expected to act similarly. The action of other acids similar to picric, as regards pK and penetrating ability, has not yet been investigated; however, acetic, boric, and tannic acids have been used in experiments similar to those performed with picric acid. Acetic acid was used in concentrations from M/6000 to M/300; boric acid, from $M/10^5$ to M/5; and tannic acid, from $M/10^6$ to M/100; the upper limits of concentrations used were factors of the solubility and the effects of the acids on the eggs. Over M/1000, acetic acid often injured the eggs irreversibly, so that they were not fertilizable; this makes it difficult to evaluate cases of inhibition by acetic acid of activation, in the absence of tests for reversal of the effect. Ten to twenty experiments were performed with each acid in attempts to demonstrate synergism with heat, in the manner of picric acid; the duration of the baths ranged from 30 minutes to 24 hours. On a few occasions, acetic acid in concentrations around M/500 (concentrations not always innocuous) showed the synergistic action, but as often acted in the opposite manner (probably because of injury to the eggs). On one batch of eggs, M/10–M/20 boric acid also showed some synergistic action with heat, but this did not recur in similar experiments with other batches of eggs.

A further corroboration of the interpretation in terms of an activator-substance was sought in several attempts to accumulate the activator more rapidly by heating the eggs in picric acid, with subsequent release to sea water. In only 3 of 16 such

experiments was there markedly more activation in the eggs so treated than in those similarly exposed to the acid without application of the heat. However, none showed differences in the other direction; no data of any experiment thus far performed militates against the suggested scheme.

The completely reversible inhibitory action of picric acid is similar to the action of isotonic citrate in the experiments of Heilbrunn and Wilbur (1937) and Wilbur (1941). Since the citrate is presumed to act by removing calcium ions from solution by the formation of calcium citrate, there is a suggestion that perhaps calcium picrate is a similarly weakly dissociated salt. However, a few measurements of the electrical resistance of calcium picrate solutions showed that the equivalent conductance increased only slightly with dilution over the range n/100–n/10,000. (The increase was in proportion to that found with $CaCl_2$ in the same concentrations; the equivalent conductance of calcium citrate increased enormously with dilution over this range of concentration.) Thus the inhibitory action of picrate cannot be explained on the same basis as that applied to citrate inhibition.

In their extensive experiments on the peculiar action of many substituted phenols on the eggs of the sea-urchin, Clowes and Krahl (1936), Krahl and Clowes (1936, 1940), and Tyler and Horowitz (1937b, 1938) found picric acid one of only two or three inactive members of this group of compounds. Inhibition of cleavage was encountered only at concentrations around M/100 or more, and the stimulation to respiration characteristic of this chemical group was lacking altogether. The calculations of Tyler and Horowitz showed that the concentration of dissociated picrate inside the cells was about 100 × that at which the related substances showed similar effectiveness. The same sort of relation was found for the other relatively inactive phenols. The latter should be tested in experiments similar to those with picric acid reported here. Such investigations might aid in deciding whether the action of picric acid is to be attributed to its acidity or to its particular molecular configuration.

Perhaps picric acid is unique in its combination of a low pK and a rapid rate of penetration into cells. The other phenols found to be exceptional (as regards inhibition of cleavage and stimulation to respiration) may share this combination of properties. Additional experimentation involving alteration of the picrate/picric acid ratio in the solution (through addition of HCl or NaOH) is also suggested; such data might strongly indicate whether the acidity or the picrate itself is the active factor. On either basis, the present data clearly show that, while anesthetizing the eggs, this active factor constantly renders them increasingly sensitive to the removal of the anesthetization, and to subsequent stimuli. Proper interpretation of this fact might lead to a significant contribution to the understanding of the nature of stimulation.

Summary

1. Germinal vesicle breakdown in *Nereis limbata* eggs, brought about by heat, or addition of KCl or sodium citrate to the sea water, was inhibited by the addition of picric acid at about M/1000.

2. After immersion for a few hours in M/1000 picric acid in sea water, germinal vesicle breakdown occurred upon application of subliminal doses of heat, KCl, or sodium citrate.

3. After immersion for 6–70 hours, removal of the eggs from picric acid to ordinary sea water caused germinal vesicle breakdown.

4. Activation by ultra-violet irradiation did not conform in these relations to picric acid, under the conditions of the experiments.

5. These results are interpreted on the basis of a hypothetical activating substance produced within the egg, and inactivated or bound by picric acid.

6. The relation of picric acid to the calcium ion and the combination of calcium with protoplasmic proteins is considered, in an alternative explanation of the results.

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